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## **Mesenchymal stromal cells for treatment of the acute respiratory distress syndrome: The beginning of the story**

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## **Bench to bedside review article for the Journal of the Intensive Care Society**

### **Title: Mesenchymal stromal cells for treatment of the Acute Respiratory Distress Syndrome: the beginning of the story**

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#### **Abstract**

In spite of decades of research, the Acute Respiratory Distress Syndrome (ARDS) continues to have an unacceptably high mortality and morbidity. Mesenchymal stromal cells (MSCs) present a promising candidate for the treatment of this condition and have demonstrated benefit in preclinical models. MSCs, which are a topic of growing interest in many inflammatory disorders, have already progressed to early phase clinical trials in ARDS. Whilst a number of their mechanisms of effect have been elucidated, a better understanding of the complex actions of these cells may pave the way for MSC modifications which might enable more effective translation into clinical practice.

#### **ARDS**

ARDS is a devastating clinical disorder with a variety of aetiologies that induces an excessive inflammatory response. Widespread damage to the alveolar compartment ensues leading to the development of hallmark traits of ARDS such as hypoxia and pulmonary oedema<sup>(1)</sup>. First described by Ashbaugh *et al.* in 1967 as the “acute onset of tachypnoea, hypoxemia, and loss of compliance”<sup>(2)</sup>, ARDS has since been studied extensively. Presently the diagnosis requires that the condition develop within one week of the underlying insult, that bilateral lung infiltrates are evident through chest imaging and that respiratory failure cannot be wholly attributed to cardiac failure and hydrostatic oedema<sup>(3)</sup>.

Epidemiological studies report that mortality and morbidity associated with ARDS remains significant; rates vary from 25-40% and are dependent on the severity of the condition<sup>(3-5)</sup>. There has been an improvement in mortality rates over time which reflect improvements in supportive care and in particular the preferential use of protective lung ventilation and other interventions to limit injurious ventilation<sup>(4, 6, 7)</sup>. An effective therapeutic intervention which targets the underlying

pathophysiology remains elusive<sup>(8)</sup> and many candidates which initially showed promise in preclinical studies have been of no clinical benefit with regards to mortality<sup>(9-13)</sup>. The heterogeneous patient population coupled with a complex pathophysiology underlying the development of ARDS may explain the difficulty of the challenge that researchers are faced with in developing a therapy for ARDS. It is arguable that a therapy which targets multiple aspects of the pathophysiology of ARDS may have greater potential to improve outcomes.

## **MSCs**

MSCs are a heterogeneous population of cells found in nearly all adult tissues including bone marrow, placenta, adipose tissue, skin and skeletal muscle<sup>(14-16)</sup>. With the capacity to differentiate into cells of both mesenchymal and non-mesenchymal lineage, the potential application in regenerative medicine has been widely recognised<sup>(17-19)</sup>.

Further study has identified additional qualities which may dramatically broaden the scope of their therapeutic use. One major obstacle for tissue and organ transplantation is rejection and careful donor-recipient matching is required. In contrast, there is evidence that MSCs have inherently low immunogenicity and are generally well tolerated even when administered in an allogeneic fashion<sup>(20, 21)</sup>.

In non-inflammatory conditions MSCs do not express MHC class II but expression is triggered by stimulation with IFN- $\gamma$ . However they appear to lack expression of CD40, CD80 and CD86, key co-stimulatory molecules required for T cell activation, which may explain recipient tolerance to these cells<sup>(22)</sup>. Conflicting evidence has been reported that MSCs are not in fact immune privileged and do elicit immune responses in non-matched hosts<sup>(23, 24)</sup>. These confounding results could be a result of the inconsistencies in the source of MSCs or perhaps the disease setting itself. Regardless, it seems that MSCs may not be universally tolerated in all non-matched patients and emphasizes the need for further study, including testing patients who receive MSCs for development of anti-HLA antibodies.

Another potential concern with using stem cells therapeutically is in their tendency to become tumorigenic as has been shown with embryonic stem cells<sup>(25)</sup>. Adipose-derived MSCs have been shown to maintain genetic stability for at least 12 passages *in vitro* and show no evidence of tumour development when given intravenously to immunodeficient mice at a range of doses<sup>(26, 27)</sup>. Bernardo *et al.* similarly show that human bone marrow-derived MSCs when cultured to passage 25 show no alteration in telomerase activity or telomere length, again suggesting genetic stability<sup>(28)</sup>. In contrast, another study showed malignant transformation of human MSCs in long term culture<sup>(29)</sup> although these cells were cultured *in vitro* for up to 105 weeks. It is likely that MSCs used therapeutically will be cultured for a much shorter period. Furthermore, MSCs have also been reported to promote breast cancer metastasis *in vitro*<sup>(30)</sup>. With conflicting data regarding their tumorigenicity, it will be imperative to have stringent quality control processes in place to monitor their safety when administered to patients, including long-term follow-up.

The immunomodulatory actions of MSCs are well documented; interactions with T lymphocytes, NK cells and dendritic cells amongst others confer them with many regulatory functions in terms of both innate and adaptive immunity<sup>(31, 32)</sup>. MSCs can inhibit T cell proliferation or promote regulatory T cells via induction of an anti-inflammatory macrophage phenotype<sup>(33, 34)</sup>. They suppress proliferation, cytokine production and cytotoxicity of NK cells towards HLA class I expressing targets and can prevent the differentiation of monocytes into dendritic cells as well as decrease the antigen presentation capacity of mature dendritic cells<sup>(35, 36)</sup>. MSCs immunosuppressive effects also prolong the survival of allogeneic grafts given in a number of settings<sup>(37, 38)</sup>.

Moreover, MSCs naturally home to sites of injury when administered intravenously. Stromal cell-derived factor-1 (SDF-1) is produced by resident cells in response to injury<sup>(39, 40)</sup>. The chemotactic receptor CXCR4, which binds SDF-1, is expressed on a subset of MSCs and provides an important mechanism in MSC homing<sup>(41)</sup>. A recent study also found SDF-1 ligation increased Akt kinase signalling and enhanced paracrine factor secretion emphasizing MSC responsiveness to physiological

cues in their environment<sup>(42)</sup>. Rolling and adhesion of MSCs along blood vessels is facilitated by P-selectin and VCAM-1 expression on endothelial cells and *in vivo* imaging has demonstrated the interaction of MSCs with platelets and neutrophils to form clusters, thereby mediating MSC trafficking to inflamed sites<sup>(43, 44)</sup>. It is important to note that MSCs are relatively large cells and have a tendency to become entrapped in small diameter vessels, with reports of sequestration to the lung microvasculature<sup>(45, 46)</sup>. Another study further investigated the *in vivo* distribution of MSCs after infusion using real time imaging. Gao *et al.* observed MSC accumulation primarily in the lungs immediately after systemic infusion with smaller numbers in the liver and spleen. By 48 hours there is a shift of MSCs from the lungs towards the liver<sup>(47)</sup>. The homing of MSCs to the lung may be pertinent to their efficacy in ARDS, as it allows targeted paracrine factor delivery.

### **MSCs in preclinical disease models of ARDS**

Given these data, MSCs have been tested in a range of preclinical models of inflammatory conditions including acute renal failure, myocardial infarction and sepsis where they were found to be of benefit<sup>(48-50)</sup>. The therapeutic potential of MSC in ARDS has been studied extensively during the past decade, using different MSC sources, treatment regimens and models of lung injury. Intrapulmonary delivery of murine bone-marrow derived MSCs into mice 4 hours after endotoxin-induced lung injury improved survival, reduced oedema and improved barrier permeability<sup>(51)</sup>. These MSCs, when given intratracheally, were also protective in a live *E.coli* pneumonia model of lung injury, given 4 hours after infection<sup>(52)</sup>. In a bleomycin-induced lung injury model in mice, intravenous administration of human umbilical cord MSCs 24 hours after injury resulted in reduced fibrosis and inflammation<sup>(53)</sup>. Rat and human MSCs also have the capacity to improve repair of the lung following ventilator-induced lung injury<sup>(54, 55)</sup>. MSCs are similarly therapeutic in larger animal models; in a sheep model of ARDS induced by smoke inhalational and bacterial pneumonia human MSCs improved oxygenation

and pulmonary oedema<sup>(56)</sup>. Providing further evidence of the potential of MSCs in patients with ARDS, Lee *et al.* developed a human *ex vivo* lung perfusion model of endotoxin-induced and live bacteria-induced injury. Allogeneic MSCs given 1 hour after injury improved barrier permeability and alveolar fluid clearance whether given intratracheally or intravenously<sup>(57, 58)</sup>.

### **Mechanisms of MSC effect in lung injury**

One of the most valuable qualities which can be attributed to MSCs, which pharmacologic therapies lack, is the ability to actively respond to the local environment. This allows MSCs to have an individual and potentially varied therapeutic effect targeting multiple aspects of ARDS. A number of mechanisms by which MSC act have been identified.

#### *Engraftment*

Considering the loss of integrity of the alveolar epithelium following injury it was hypothesized that MSCs, with their pluripotency, may engraft into the epithelium and so contribute to regeneration. Whilst engraftment of MSCs into the lung epithelium is documented, it appears to be a rare event in the context of lung injury with reports of less than 5% engraftment occurring and so it is likely this does not represent the primary mechanism of their effect<sup>(51, 59-63)</sup>.

#### *Paracrine factors*

Given the seemingly low capacity for engraftment into the alveolar epithelium, it is generally considered that the secretion of paracrine factors is one of the primary mechanisms of their effect. A number of groups show that keratinocyte growth factor (KGF), produced by MSCs, is essential for the restoration of alveolar epithelium permeability and alveolar fluid clearance after injury by rescuing the activity of the sodium channel ENaC<sup>(57, 64, 65)</sup>. Similarly, angiopoietin-1 was found to be responsible for the MSC protective effects on type II alveolar epithelial cell permeability in an *in vitro* model<sup>(66)</sup>.

One worry with a therapy having anti-inflammatory effects being utilized in an infectious setting, as is often the case in ARDS, is that the host's ability to combat infection may be hindered. Interestingly however, MSC administration in models of sepsis and ARDS triggered by live bacteria consistently results in improved bacterial clearance despite reduced inflammation. The antimicrobial effect of MSCs is partially explained by their ability to enhance phagocytosis by cells of the innate immune system. In two different murine sepsis models, MSCs were found to increase phagocytic capacity of CD11b positive cells and blood monocytes<sup>(48, 67)</sup>. Neutrophils also demonstrate increased phagocytic activity with the influence of MSC, as seen by Hall *et al.* in a cecal ligation and puncture model of sepsis. Moreover, the depletion of neutrophils from these mice abrogated the beneficial effect of MSCs<sup>(68)</sup>. MSC-derived KGF reduced bacterial load in the *ex vivo* perfused human lung injured with *E. coli* which was associated with increased phagocytosis by alveolar macrophages, potentially by the upregulation of GM-CSF in the BALF. *In vitro* experiments, also showed a pro-survival effect of MSC-derived KGF on human monocytes<sup>(58)</sup>. Additional antimicrobial activity is exerted by MSCs through the secretion of antimicrobial peptides and proteins such as human cathelicidin, LL-37 and lipocalin-2 which binds the bacterial siderophore responsible for iron uptake, an essential micronutrient for bacterial growth<sup>(52, 69)</sup>.

TNF stimulated gene protein-6 (TSG6) is a major contributor to the immunomodulatory effects of MSCs and contributes to their benefit in a variety of conditions including myocardial infarction and wound healing<sup>(45, 70)</sup>. This is also true of the MSC effect in lung injury. In an LPS-induced lung injury model MSCs significantly upregulate TSG6 production and the blockage of TSG6 by silencing RNA resulted in near complete reversal of their anti-inflammatory effects<sup>(71)</sup>. Intriguingly, MSCs placed on nonadherent surfaces undergo compaction into spheroid aggregates. This triggers caspase-dependent IL-1 signalling in MSCs subsequently augmenting the production of TSG6 in combination with other anti-inflammatory agents<sup>(72)</sup>. The generation of these structures perhaps reflects what occurs in the pulmonary microvasculature and could partly explain their potent effects in lung injury models.

MSCs also produce anti-inflammatory cytokines which partly contribute to the decreased inflammation in preclinical models. IL-1 receptor antagonist is produced by a subset of MSCs and was found in bleomycin-induced lung injury to prevent the upregulation of IL-1 $\alpha$  and TNF $\alpha$ , two key inflammatory mediators in the lung<sup>(73)</sup>. Prostaglandin E2 is a factor commonly associated with the MSC immunomodulatory effect and has been demonstrated to influence macrophages to increase production of anti-inflammatory IL-10 in a cecal ligation and puncture model of sepsis<sup>(74)</sup>.

It is important to note that whilst MSCs secrete an extensive range of anti-inflammatory cytokines, they are also capable of pro-inflammatory cytokine production in response to certain cues. IL-6 and IL-8 are secreted by MSCs, both of which have been associated with poorer outcomes in patients with ARDS<sup>(75, 76)</sup>. IL-6 is often implicated in pro-inflammatory responses however it is apparent that this cytokine is promiscuous in its functions<sup>(77-79)</sup>. Perhaps surprisingly, the therapeutic effects of murine MSCs from adipose tissue in an endotoxin-induced lung injury model were diminished with IL-6 interference<sup>(80)</sup>. Whilst the role of MSC-derived IL-8 in lung injury is not clear, there is evidence that IL-8 is able to promote VEGF production by MSCs thereby supporting a pro-angiogenic effect<sup>(81)</sup>. It is plausible that increased VEGF levels could provide a protective effect on the microvasculature in lung injury given the pro-survival influences that it exerts on endothelial cells<sup>(82, 83)</sup>. It could also be argued that the potential for MSC-derived IL-8 to promote neutrophil recruitment in lung injury is abrogated by their concomitant production of TSG6, which is known to directly bind IL-8 subsequently blocking this function<sup>(84)</sup>.

#### *Nanotubule formation and microvesicle secretion*

MSCs are capable of secreting microvesicles, small membranous compartments containing bioactive molecules<sup>(85, 86)</sup>. MSC-derived microvesicles alone were capable of attenuating *E. coli* induced lung injury in mice and recapitulating many of the therapeutic effects of the cells themselves, including



decreases in pulmonary oedema and inflammation. mRNA coding KGF contained within these vesicles was partially responsible for this phenomenon<sup>(87)</sup>.

Interestingly, *in vivo* imaging depicts the formation of connexin-43 based gap junctions between MSCs and alveolar epithelial cells allowing the transport of mitochondria to the epithelia via microvesicles. The resultant increase in ATP levels concomitantly resulted in restoration of surfactant secretion by type II pneumocytes, reduced alveolar permeability and mortality in an LPS injury model<sup>(88)</sup>. Another group also observed mitochondrial intercellular trafficking from MSCs to epithelial cells with the use of tunnelling nanotubules which was regulated by the Rho-GTPase Miro1<sup>(89)</sup>. Although the mechanisms of effect of MSCs in the context of lung injury continue to be defined, it is already apparent that their actions are multifaceted, impacting on the numerous components of the pathophysiology of ARDS.

### **MSCs in clinical trials of ARDS**

Following the success in preclinical studies MSCs have progressed rapidly to be tested in the clinic, with widespread study in diseases including steroid resistant acute graft versus host disease, Crohn's disease and vascular disease<sup>(90-99)</sup>. To date, studies investigating MSCs in ARDS has been primarily concerned with safety and feasibility of their delivery to patients<sup>(100)</sup>. A randomized, placebo controlled pilot study carried out with the use of allogeneic adipose-derived MSCs ( $1 \times 10^6$  cells/kg of body weight, cells at passage of up to six) in patients with ARDS (defined by a PF ratio < 200mmHg) suggested that the treatment was not associated with any acute safety issues<sup>(101)</sup>. There were no differences in duration of hospital stay, ventilator or ICU-free days, although the study was not powered for these clinical outcomes. There was a decrease in serum surfactant protein-D levels (a biomarker for type II alveolar epithelium injury/activation) although the significance of these data are unclear.

A further recent multicentre, open-label, dose-escalation study sought to determine the safety and feasibility of administration of allogeneic bone-marrow derived MSCs in patients with moderate-to-

severe ARDS (defined as a PF ratio < 200mmHg receiving PEEP > 8cm H<sub>2</sub>O). The MSCs used here were at passage 2 and were administered in three doses; low dose (1x10<sup>6</sup> cells/kg), intermediate dose (5x10<sup>6</sup> cells/kg) and high dose (10x10<sup>6</sup> cells/kg). It was concluded that there were again no acute MSC-related adverse events in the study<sup>(102)</sup>. The significance of the findings in these two studies are limited by the small patient numbers (12 and 9 respectively) and short follow-up but certainly justify progression to phase II clinical trials, which are currently underway (clinicaltrials.gov, NCT02097641). The long term effects of MSC treatment in patients of ARDS remain to be defined.

It is important to note that in these studies only a single dose of MSCs was examined, with safety being the primary outcome. This is in contrast to trials in other diseases where multiple doses were given over an extended period of time (e.g. once weekly over four weeks in the case of Crohn's disease<sup>(93)</sup> or twice weekly over four weeks for graft-versus-host-disease<sup>(98)</sup>) and were also found to be well tolerated and in some cases potentially efficacious in providing a therapeutic effect. Whilst it is possible that repeated doses of MSCs could be safe and more effective in ARDS, it is unwise to infer this based on findings from conditions so significantly different. Certainly, larger phase II studies elucidating the safety and efficacy of a single dose of MSCs are required before multiple dosing regimens should be investigated.

### **Optimisation of the therapeutic effect of MSCs**

Preclinical research studying MSCs as a treatment modality is ongoing and there is a significant effort to maximise their effects. Numerous factors appear to have effects on the efficacy of MSCs in practice. For example, it is recognised that the route of administration can influence their benefit with intraperitoneal delivery more effective than intranasal in neonatal lung injury<sup>(60)</sup>. This is further evidenced by the observation that intraperitoneal injection of MSCs is inferior to intratracheal or intravenous application in ventilator-induced lung injury<sup>(55)</sup>. Another challenging task is to verify the optimal tissue source of MSCs. MSCs from different niches have distinctive attributes associated with them which may confer advantages depending on the context<sup>(103-105)</sup>. For example, comparison

of human bone marrow, adipose and umbilical cord MSCs shows that umbilical cord MSCs have higher proliferative rates and lower expression of senescence markers such as p53 and p21<sup>(104)</sup>. This could suggest that umbilical cord MSCs would be more beneficial in regenerative applications.

The *ex vivo* expansion of human MSCs and conditions they are exposed to prior to use has profound effects on their phenotype. Several studies have identified that maintenance of MSCs in hypoxic conditions alter MSC activity. Hypoxia, a characteristic feature of ARDS, is associated with heightened chemotaxis and cell viability coupled with upregulated secretion of paracrine factors<sup>(106)</sup>. Hypoxic exposure of MSCs results in a higher proportion of self-renewing cells with a more homogenous population compared to cells maintained in normoxic conditions<sup>(107)</sup>. Intriguingly, preconditioning MSCs in serum from ARDS patients before treating endotoxin-injured mice elicited a more potent IL-10 and IL-1ra response and consequentially improved outcomes<sup>(108)</sup>. Preconditioning of MSCs in patient serum was also implemented by Zheng *et al.* in their adipose-tissue MSC clinical trial in ARDS patients<sup>(101)</sup>.

An understanding of how the local environment modifies MSC function has highlighted how MSCs might be manipulated to perhaps amplify their effects. Overexpression of soluble IL-1 receptor-like-1 in MSCs, which competes with transmembrane IL-1 receptor-like-1, for IL-33 ligation, markedly increased the anti-inflammatory and reparative effects of these cells in endotoxin-induced lung injury compared to standard MSCs<sup>(109)</sup>. IL-33 is expressed constitutively in the nuclei of epithelial and endothelial cells in many human tissues including the lung and is released upon damage. IL-33 is then able to elicit inflammatory responses<sup>(110, 111)</sup>. MSCs transfected with a vector overexpressing angiopoietin-1 reduced inflammation and permeability to a greater extent in an LPS model of lung injury compared to control MSCs<sup>(112)</sup>. MSC engraftment into the lung can be augmented through the blockage of the Wnt/ $\beta$ -catenin signalling pathway which normally acts to induce differentiation towards a fibroblast or myofibroblast phenotype<sup>(113)</sup>.

Another element regulating MSC function and phenotype is TLR stimulation. Waterman *et al.* described the induction of a pro-inflammatory MSC and immunosuppressive MSC phenotypes resulting from TLR4 and TLR3 stimulation respectively<sup>(114)</sup>. TLR4-stimulated MSCs produced higher levels of pro-inflammatory IL-6 and IL-8, whereas TLR3 stimulation enhanced secretion of anti-inflammatory IL-4 and IL-1ra. A conflicting study demonstrates that TLR3 stimulation resulted in the highest induction of IL-6 and IL-8 but the MSC sources between these studies differed<sup>(115)</sup>. Moreover, the priming of bone marrow-derived MSCs with TLR3 and not TLR4 afforded the cells with increased resistance to NK cell killing as well as amplifying their immunosuppressive effects on these cells<sup>(116)</sup>.

Ongoing research in MSC therapy is crucial to uncover how the biological effects of MSCs can be potentiated to enable transition of MSCs to the bedside as the optimal treatment for patients with ARDS.

### **MSC isolation and expansion quality control**

One of the major disadvantages limiting the progression of this cell therapy to the clinic is the poor characterisation of these cells in combination with the heterogeneity of their therapeutic effects. The European Medicines Agency and British Standards Institution both highlight the need for detailed profiling of MSCs, improved isolation and purification procedures and understanding of their mechanisms of action. The importance of MSC source and culture method is emphasized in comparative studies demonstrating substantial contrast in their immunomodulatory functions. For example, the use of foetal calf serum or platelet lysate for their expansion has significant effects on their abilities to inhibit T-cell growth<sup>(117)</sup>. Other studies underline other key factors influencing MSC biology including the age of the donor or the levels of serum or glucose used in culture<sup>(118-120)</sup>.

As a result, there is significant work being carried out to create optimized methods of isolation and handling of MSCs<sup>(117, 121-124)</sup>. Mimicking the bone marrow ECM which the MSCs inhabit *in vivo* results in improvements in their stemness and proliferative capacity<sup>(125)</sup>. Furthermore, Carrancio and

colleagues, when modifying a number of culture conditions for expansion, reported that platelet lysate supplementation and hypoxia resulted in the largest yields of MSCs expanded *ex vivo*<sup>(126)</sup>.

The inconsistency of the MSC effect in certain conditions could be attributed to any number of these variables related to their source, isolation and expansion. An emerging line of research, therefore, is the development of potency assays for MSCs which could be used before administration to patients. Expression of TSG6 appears to correlate with their anti-inflammatory potency in a model of chemical injury to the cornea but conversely its expression was negatively associated with osteogenic differentiation capacity<sup>(127)</sup>. Use of a combination of simple *in vitro* assays enabled the efficacy of bone marrow-derived MSCs in treatment of murine wounds to be defined<sup>(128)</sup>. These assays determined MSC growth, proliferation and viability using cell counts, bromodeoxyuridine incorporation and measurement of cellular ATP levels respectively. Higher scoring in these assays was associated with more extensive engraftment into the wound site.

It is therefore critical that isolation, expansion and screening procedures for MSCs in the treatment of lung injury be optimized and standardized.

## **Summary**

There remains urgent need for an effective therapy for ARDS. MSCs are a highly versatile cell population with potential implications as a treatment for ARDS based on their immunomodulatory capacity, reparative properties, ease of isolation and propagation and the feasibility as an allogeneic therapy. A wealth of evidence supports the case for MSC based therapy in patients with ARDS and has paved the way for ongoing clinical trials. However much remains to be defined about the role of MSCs in ARDS. The importance of further investigation of MSCs cannot be overstated and is necessary to determine the most appropriate application of MSCs and optimising their therapeutic effects in ARDS.

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